FEBS Letters 582 (2008) 1508–1513

Molybdate transport through the plant sulfate transporter SHST1

Kate L. Fitzpatrick, Stephen D. Tyerman, Brent N. Kaiser*

School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus Urrbrae, SA 5064, Australia

Received 25 January 2008; revised 19 March 2008; accepted 26 March 2008

Available online 7 April 2008

Edited by Ulf-Ingo Flügge

Abstract **Molybdenum is an essential micronutrient required by plants. The mechanism of molybdenum uptake in plants is poorly understood, however, evidence has suggested that sulfate transporters may be involved. The sulfate transporter from *Stylosanthes hamata*, SHST1, restored growth of the sulfate transport yeast mutant, YSD1, on media containing low amounts of molybdate. Kinetic analysis using $^{99}MoO_4^{2-}$ demonstrated that SHST1 enhanced the uptake of molybdate into yeast cells at nM concentrations. Uptake was not inhibited by sulfate, but sulfate transport via SHST1 was reduced with molybdate. These results are the first measurement of molybdate transport by a characterised plant sulfate transport protein.**

Keywords: Molybdate transport; Molybdenum; Sulfate transport; SHST1; YSD1

1. Introduction

Molybdenum (Mo) is essential for plant growth and is a rare element with a crustal abundance of 1.2 mg/kg [1]. Availability of the soluble oxyanion, molybdate (MoO_4^{2-}), decreases with increasing acidity and/or in soils rich in iron oxides [2]. However, for soils above pH 4.23 and those rich in organic matter, molybdate becomes the predominate available form [1]. Molybdate is active within the plant when complexed by the pterin compound named molybdenum cofactor (Moco). Only a few plant enzymes interact with Moco; nitrate reductase (NR), aldehyde oxidase (AO), xanthine dehydrogenase (XDH) and sulfite oxidase (SOX) where Mo participates as a transition metal in reduction/oxidation reactions [3]. When Mo is deficient striking phenotypes can develop, including nitrogen starvation responses, stem and leaf development disorders (e.g. whiptail in crucifers), leaf necrosis, and reduced fruit set [4].

Molybdate transport involves the ModABC system in prokaryotic systems [5] while in eukaryotes the transport mechanism has recently been characterised involving a class of transport proteins called MOT1 [6–8]. MOT1 is a relative of the sulfate transporter superfamily [9] but does not appear to transport sulfate. The role of MOT1 in plant molybdate uptake is still unclear as recent results suggest MOT1 is localised to mitochondria [8]. In plants, the uptake of molybdate may occur through sulfate transport proteins [10,11], as both molybdate and sulfate have similar chemical properties [12]. In plants, sulfur (S) starvation can enhance Mo accumulation [13] or alternatively repress Mo uptake when supplied at increasing concentrations [14].

The *Stylosanthes hamata* sulfate transporter SHST1 is expressed in roots and is enhanced under S starvation [15]. When expressed in YSD1 yeast, SHST1, can accumulate sulfate and is capable of rescuing growth when sulfate concentrations in the media are low [15]. SHST1 is a high affinity H^+/SO_4^{2-} cotransporter with a K_m for sulfate of 10 μM.

We have re-examined the functional transport properties of SHST1 in the yeast mutant YSD1 in the context of its ability to also transport molybdate.

2. Materials and methods

2.1. Yeast media and growth

YSD1 (*sul1 his3-Δ1 leu2 trp1-289 ura3-5*) was transformed with SHST1 (in pYES3 [15]) and empty vector controls (pYES3) using LiAc/PEG [15]. Transformed cells were grown on media containing either low levels of Mo (LMB) ([Mo] was below detection by ICP-MS, approximately less than 0.4 nM) or low levels of sulfate (LSB) [16]. To these media additions were made of sulfate, 76.52 mg/l homocysteine thiolactone (TL), or molybdate as indicated. LMB media consisted of a modified Grenson's media [17] where $Na_2MoO_4 \cdot 2H_2O$ was omitted (normally 16.5 nM) and solutions prepared using Mo scrubbed dH_2O [18].

2.2. $^{99}MoO_4^{2-}$ and $^{35}SO_4^{2-}$ uptake assays

Cells used in uptake studies were grown initially in standard liquid SC glucose media to an $OD_{600\,nm}$ of 1.0. Cells were then grown as indicated in either LMB + 2% (w/v) galactose or LSB + TL + 2% (w/v) galactose. All cultures were grown with constant shaking (200 rpm) at 28 °C. At mid-log phase, cells were washed and resuspended to an $OD_{600\,nm}$ of 5 in a base 20 mM KPO_4 reaction buffer (pH 5.6 for $^{35}SO_4^{2-}$ and pH 6.5 for $^{99}MoO_4^{2-}$) containing 2% (w/v) galactose. Yeast uptake assays consisted of taking 50–100 μl of cell culture in reaction buffer and shaking in a 2 ml round bottomed microfuge tube with equal amounts of labelled $^{99}MoO_4^{2-}$ (as $Na_2^{99}MoO_4^{2-}$; ANSTO-ARI) or $^{35}SO_4^{2-}$ (as $Na^{35}SO_4^{2-}$; GE Healthcare) in 20 mM KPO_4 buffer with pH modifications and added anions as indicated. At indicted time points, 50–100 μl of the cell/buffer mix were harvested and placed in 5 ml ice-cold non-radioactive reaction buffer and filtered by vacuum onto 0.45 μM nitrocellulose filters (Millipore). Harvested cells were washed with 10 ml of ice-cold non-radioactive reaction buffer before being placed into 4 ml of aqueous scintillant (Perkin–Elmer) and radioactivity measured in a scintillation counter (Beckmann). Protein determinations were performed by TCA precipitation [19].

3. Results

3.1. SHST1 enhances growth of YSD1 on low Mo containing media and enhances the uptake of $^{99}MoO_4^{2-}$

We developed a low Mo yeast media (LMB) to characterise the transport properties of SHST1 in YSD1. When we plated

*Corresponding author. Fax: +61 08 8303 7116.
E-mail address: brent.kaiser@adelaide.edu.au (B.N. Kaiser).

0014-5793/$34.00
doi:10.1016/j.febslet.2008.03.045

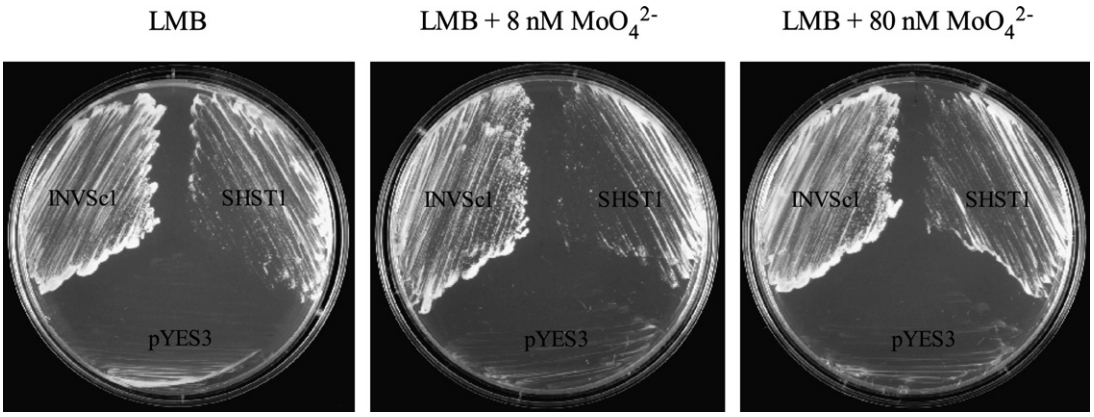

Fig. 1. Growth of YSD1 and wild type cells transformed with pYES3 or SHST1 on low concentrations of molybdate. Cells were plated onto LMB media containing increasing concentrations of MoO_4^{2-} with 2% (w/v) galactose (Gal). pYES3 = pYES3/YSD1, SHST1 = pYES3/SHST1/YSD1, INVSc1 = pYES3/INVSc1.

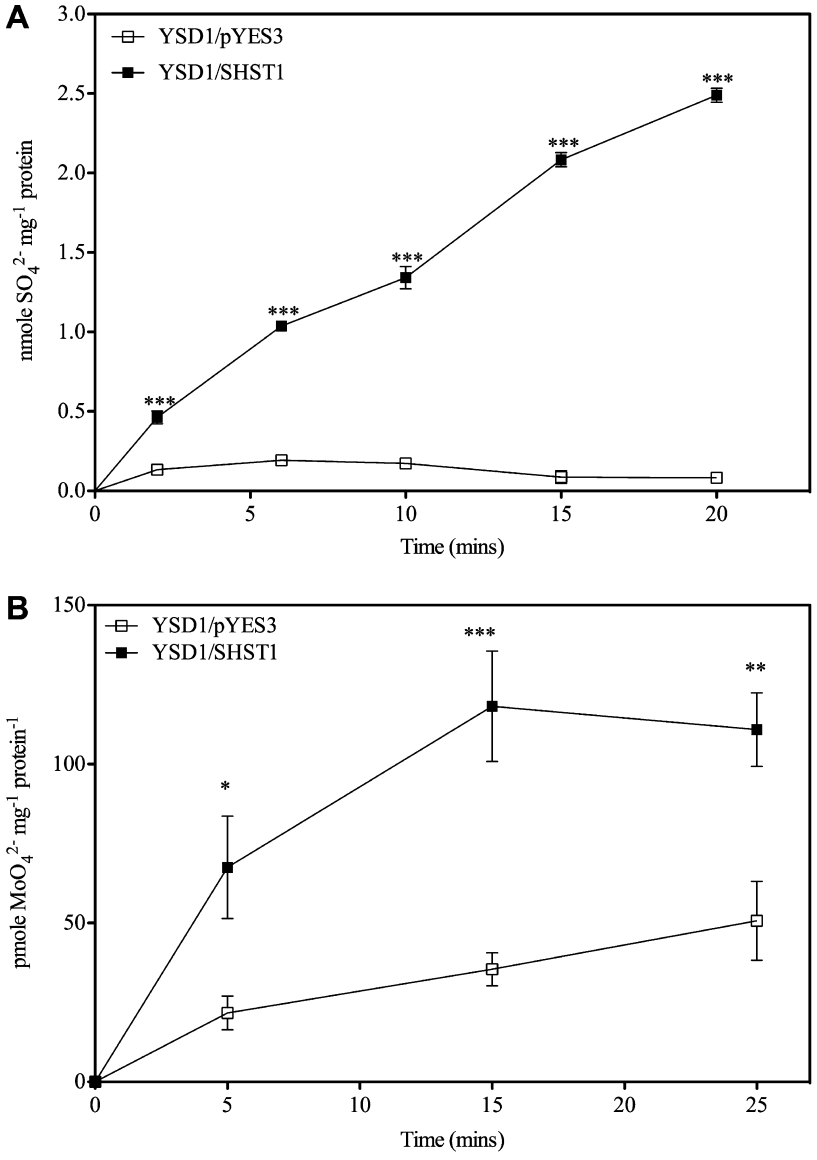

Fig. 2. Accumulation of $^{35}SO_4^{2-}$ or $^{99}MoO_4^{2-}$ in YSD1 cells containing SHST1 or the empty vector control pYES3. (A) Uptake of $^{35}SO_4^{2-}$ over time from an external concentration of 25 μM in transformed cells grown in LMB. (B) Uptake of $^{99}MoO_4^{2-}$ (10 nM external concentration) over time in transformed cells grown initially in LMB. Values are means ± S.E.M. ($n = 4$) and is representative of three independent experiments. Data points with *, **, *** are significantly different from controls at $P < 0.05$, 0.01, 0.001, respectively.

YSD1 containing pYES3 onto LMB media, cells grew slowly (Fig. 1). In contrast improved growth was observed with both the wild type (INVSc1: pYES3) and YSD1 cells transformed with SHST1. When the LMB media was supplemented with 8 or 80 nM MoO_4^{2-}, SHST1 growth improved further, while the empty vector controls continued to grow slowly.

Heterologous expression of SHST1 in YSD1 cells was confirmed by measuring the uptake of 25 μM $^{35}SO_4^{2-}$ over a defined time period (Fig. 2A). As expected, SHST1 accumulated significantly higher levels of sulfate over that of the controls. Cells were then exposed to 10 nM $^{99}MoO_4^{2-}$ in a similar time course experiment. SHST1 significantly enhanced the uptake of $^{99}MoO_4^{2-}$ relative to the empty vector YSD1 controls (Fig. 2B) where after a 15-min exposure, SHST1 had accumulated a three-fold higher level of $^{99}MoO_4^{2-}$ over the controls. $^{99}MoO_4^{2-}$ was then supplied at increasing concentrations up to 1000 nM (Fig. 3A). Rate of $^{99}MoO_4^{2-}$ influx was linear where SHST1 was about 2.8-fold higher than the pYES3 control (slope: 0.594 ± 0.012 versus 0.212 ± 0.008 pmol MoO_4^{2-} mg^{-1} protein min^{-1}/nM MoO_4^{2-}, respectively). $^{35}SO_4^{2-}$ influx by SHST1 across a similar concentration range was also found to be linear (Fig. 3B). For SO_4^{2-} influx the difference between SHST1 and pYES3 controls was greater and the uptake rates 20-fold higher than that of $^{99}MoO_4^{2-}$ influx (Fig. 3B). Note that

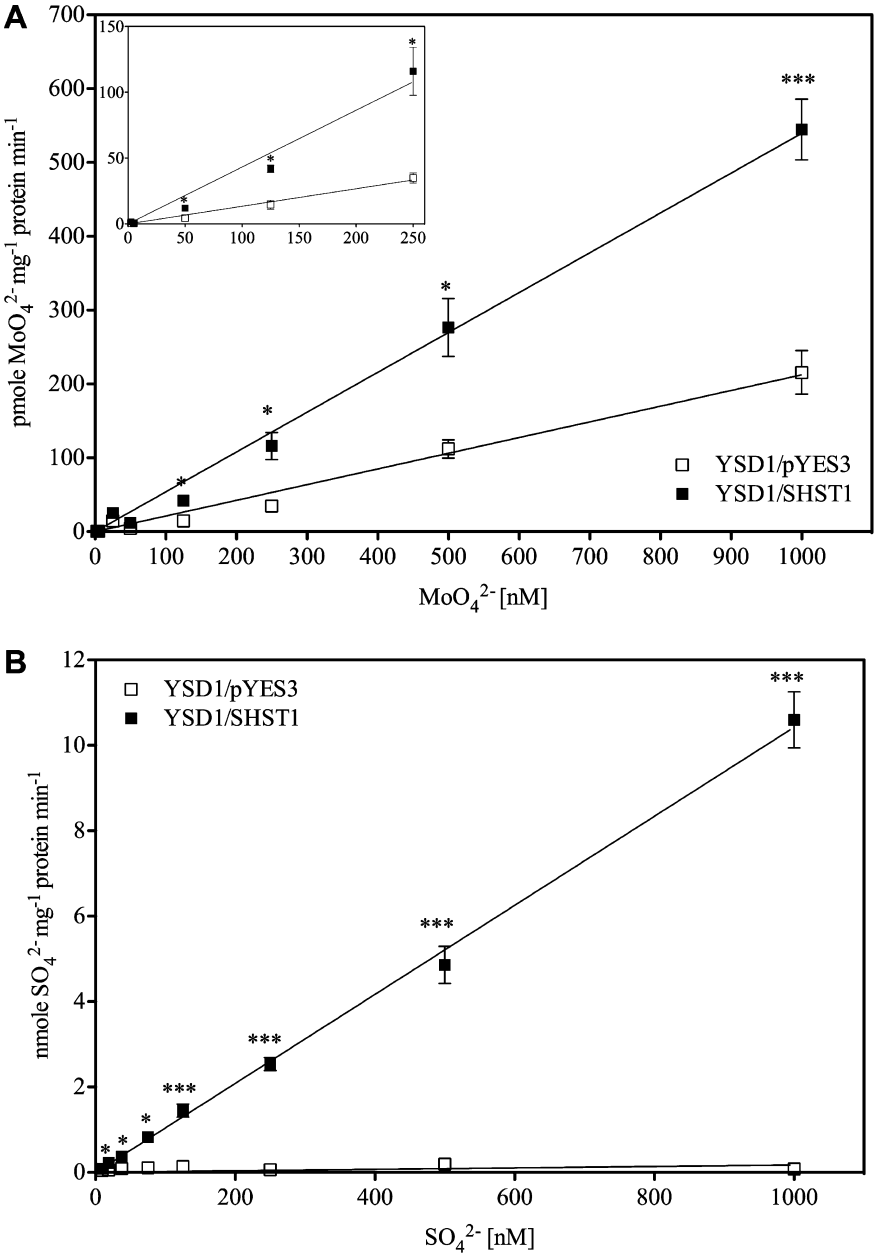

Fig. 3. Concentration dependence of $^{99}MoO_4^{2-}$ and $^{35}SO_4^{2-}$ influxes in YSD1 cells containing SHST1 or pYES3. (A) $^{99}MoO_4^{2-}$ influx of cells incubated with 0–1000 nM external Mo. The influx rate was determined from 10 min uptakes. (B) $^{35}SO_4^{2-}$ influx of cells incubated with 0–1000 nM external SO_4^{2-}. Values are means ± S.E.M. ($n = 4$) representative of three independent experiments. Data points with *, **, *** are significantly different from controls at $P < 0.05$, 0.01, 0.001, respectively.

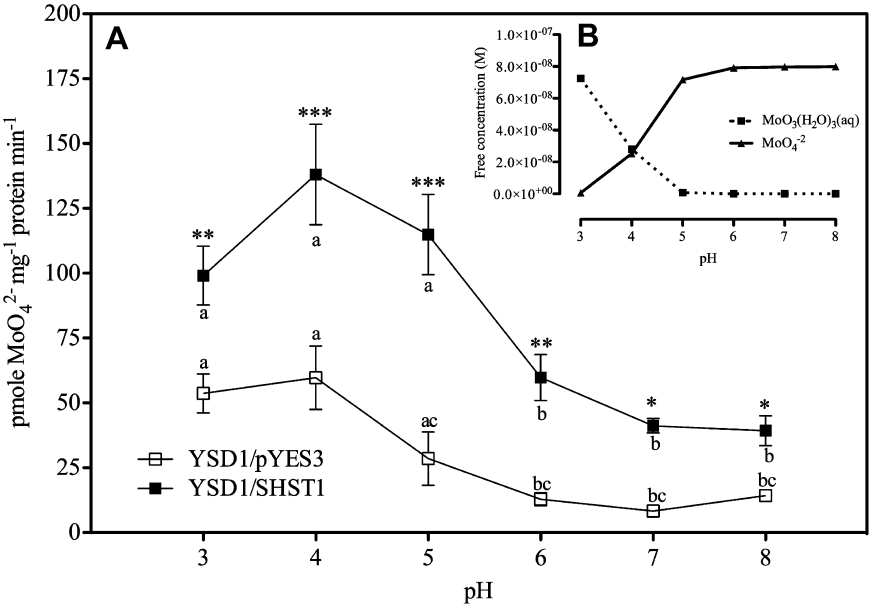

Fig. 4. Influence of external pH on the $^{99}MoO_4^{2-}$ influx. Cells were harvested and washed in sterile Mo scrubbed dH_2O and incubated with $^{99}MoO_4^{2-}$ diluted in KPO_4 buffer (pH 3–8). After 10 min cells were washed with KPO_4 buffer and the $^{99}MoO_4^{2-}$ influx rate (from 80 nM MoO_4^{2-}) determined. (A) Values are means ± S.E.M. ($n = 4$). Data points with *, **, *** are significantly different from the controls at $P < 0.05$, 0.01, 0.001, respectively. Across the pH profile of each strain, data points with similar letters are not significantly different at $P < 0.05$. (B) Inset highlights the predicted speciation of MoO_4^{2-} across the pH range 3–8.

$^{35}SO_4^{2-}$ influx showed saturation with external SO_4^{2-} concentration when examined over a higher concentration range (1–50 μM) (S1).

The pH of the external media influenced $^{99}MoO_4^{2-}$ influx in YSD1 cells containing SHST1 (Fig. 4). At pHs between 3 and 5 SHST1 elicited significantly higher ^{99}Mo uptake compared to higher pHs (6–8), but there was no significant difference between the influxes at pH 3, 4 and 5, nor between pH 6, 7 and 8. Molybdenum speciation calculations (S2) predicted that between pH 3 and 4 the predominant Mo species will be the aqueous $^{99}MoO_3(H_2O)_3$ instead of $^{99}MoO_4^{2-}$ (Fig. 4, insert B). However at pH 5, 99% of the Mo is in the form of the divalent anion and at this pH $^{99}MoO_4^{2-}$ influx was significantly higher than at higher pHs.

We examined the transport of $^{99}MoO_4^{2-}$ or $^{35}SO_4^{2-}$ when challenged with a competing anion. $^{99}MoO_4^{2-}$ transport by SHST1 was not reduced by SO_4^{2-}, WO_4^{2-}, or NO_3^- at equal (80 nM) or at 10-fold excess (800 nM) concentrations (Fig. 5A). In contrast, $^{35}SO_4^{2-}$ influx (25 μM) was reduced by approximately 48% by an equal concentration of molybdate (Fig. 5B). Molybdate was a poor competitive inhibitor of $^{35}SO_4^{2-}$ influx with a calculated K_I of 34 ± 9 μM (S1).

4. Discussion

The plant sulfate transport protein SHST1 is able to enhance the uptake of molybdate when expressed in the *Saccharomyces cerevisiae* sulfate transport mutant YSD1. SHST1 rescued growth of YSD1 on low concentrations of Mo (80 nM) and using the radioactive tracer, $^{99}MoO_4^{2-}$, SHST1 accumulated $^{99}MoO_4^{2-}$ in excess of controls. Kinetic analysis revealed a non-saturating $^{99}MoO_4^{2-}$ influx across a physiological relevant range of molybdate concentrations (0–1000 nM). This was similar to that of $^{35}SO_4^{2-}$ influx over the same concentration range. Based on the similar sizes of the sulfate and molybdate metal–O lengths, net charge, hydrogen bonding properties and tetrahedral geometry [12], we presumed sulfate would be an effective competitor to molybdate influx if both were transported by the same protein. Surprisingly sulfate failed to compete with molybdate influx when supplied at equal or at 10-fold higher concentration. This is relevant to the potential function of SHST1 as a Mo uptake system in plants where sulfate would normally be at a much higher concentration than Mo. These results are consistent with the recent characterisation of MOT1 in both Arabidopsis and *Chlamydomonas reinhardtii* where sulfate failed to compete with Mo uptake when MOT1 was expressed in yeast cells [6,7]. However, the lack of a response to sulfate contradicts previous studies in plants, which have shown that excess sulfate can inhibit Mo uptake [14]. From our results direct competition of molybdate uptake by sulfate ions appears to be minimal. It is possible that previously observed inhibition of Mo uptake in response to elevated levels of S is due to regulated expression and activity of the sulfate transport and assimilatory pathways rather than through direct competition. Alternatively other sulfate transporters may behave different to SHST1. In contrast, MoO_4^{2-} acted as a competitor to $^{35}SO_4^{2-}$ uptake by SHST1. This characteristic also lends weight to a dual function of SHST1, since MoO_4^{2-} would rarely attain such a high free concentration (>34 μM) in soil to the extent that it would inhibit SO_4^{2-} uptake.

SHST1 was originally characterised as a H^+/SO_4^{2-} cotransporter [15]. Analysis of $^{99}MoO_4^{2-}$ uptake at different external pH showed that SHST1 prefers a more acidic external environment. Although the change in the predominant form of Mo to a neutral species $MoO_3(H_2O)_3$ occurs at low pH this did not correlate with the higher influx observed at pH 5 where 99% of the Mo occurs as the divalent anion MoO_4^{2-}. At pH 4 and

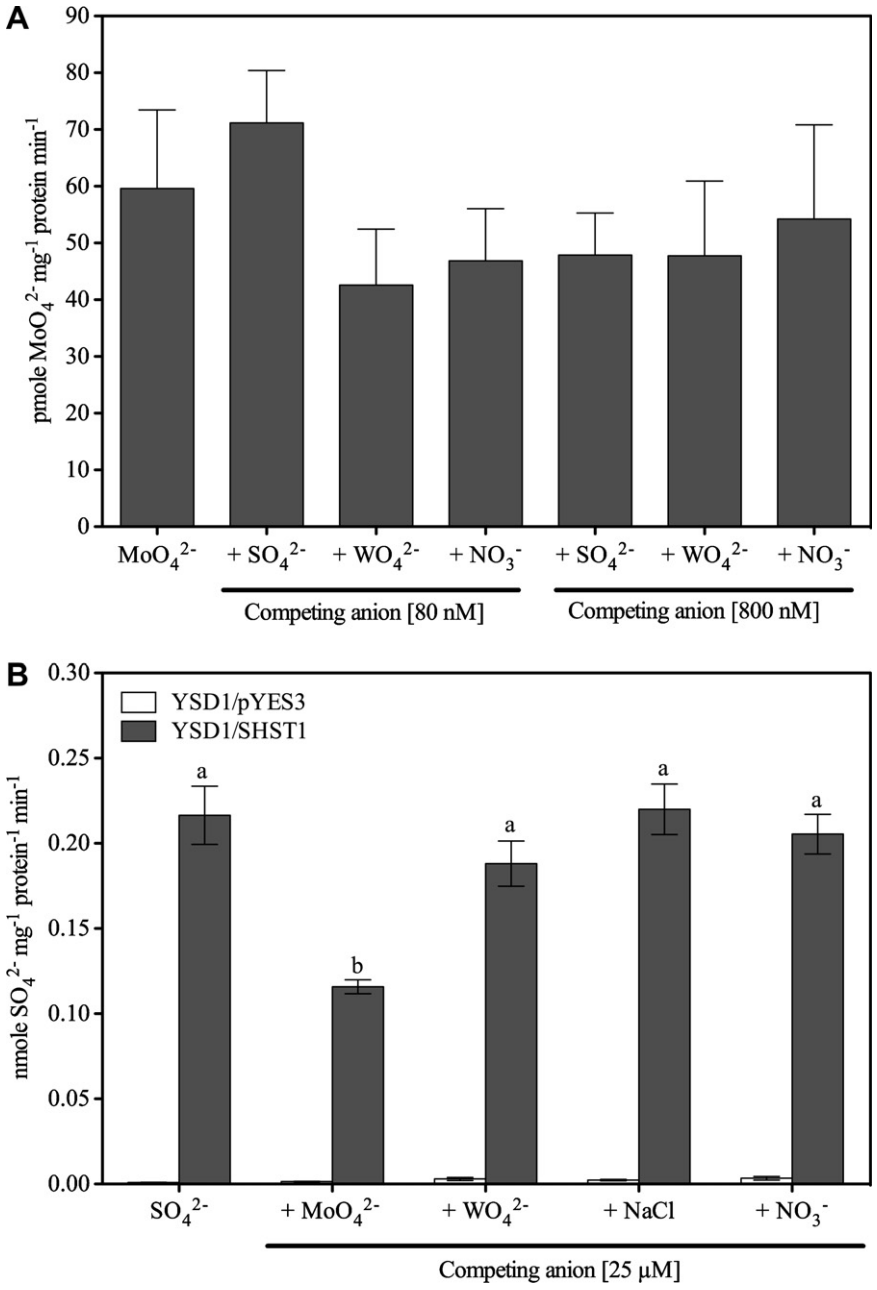

Fig. 5. Competitive effects of anions on $^{99}MoO_4^{2-}$ and $^{35}SO_4^{2-}$ influxes. Cells were grown in either LMB + Gal media (A) or LSB + TL + Gal media (B) to induce gene expression and then incubated with either 80 nM $^{99}MoO_4^{2-}$ (A) or 25 μM $^{35}SO_4^{2-}$ (B) with or without competing anions. (A) $^{99}MoO_4^{2-}$ influx into empty vector controls (pYES3 transformed YSD1 cells) was subtracted from the SHST1 influx. Data represents the combined mean ± S.E. of two independent experiments ($n = 10$). (B) Data presented is the mean ± S.E. ($n = 4$) and is representative of three independent experiments.

below more of the influx could be carried by the neutral species. As the pH was increased from 5 to 6 the uptake of MoO_4^{2-} decreased significantly which is consistent with the characterisation of SHST1 as a H^+/anion cotransporter. As a divalent anion it would be expected that uptake against a negative internal membrane potential would require active transport and the pH effect is consistent with H^+/MoO_4^{2-} cotransport.

The ability of SHST1 to transport both molybdate and sulfate may provide a functional explanation for interactions between molybdate and sulfate in prokaryotes [5] and eukaryotes [10,20,21]. In *Escherichia coli*, when the *modABC* system is inactive, molybdate uptake is thought to involve an ABC-type sulfate transporter involving a sulfate binding protein or through a non-specific anion transporter [5]. In *Penicillium notatum* [20] and *C. reinhardtii* [22], sulfate starvation will enhance the rate of molybdate transport while in *P. notatum* molybdate has been shown to be an effective inhibitor of sulfate uptake [23]. Similarly, in animal systems sulfate transport through the placental specific Na^+ coupled sulfate transporter NaS2 is competitively inhibited in the presence of molybdate [21,24]. In plants, the interaction between sulfate and molybdate

transport has been characterised in tomato [25], and rice [26]. In tomato, the translocation of $^{99}MoO_4^{2-}$ from the root to the shoot is reduced in the presence of external sulfate, however, uptake into roots did not appear to be influenced by sulfate [25]. In rice seedlings, sulfate was shown to reduce the net uptake of molybdate into roots [26]. The activity of SHST1 is different to that of MOT1 recently identified in both Arabidopsis [6] and *C. reinhardtii* [7] which does not appear to behave as a typical sulfate transporter per se as it lacks the ability to complement a yeast sulfate transport mutant, although it does allow for the uptake of MoO_4^{2-}.

In summary, the data presented here suggests sulfate transport proteins are capable of molybdate transport and provides a functional basis for observations in the literature showing strong relationships between molybdate and sulfate transport. All the characteristics of the SHST1 transporter when expressed in yeast, particularly in relation to interactions between MoO_4^{2-} and SO_4^{2-}, would indicate that SHST1 could transport both MoO_4^{2-} and SO_4^{2-} at normal concentrations of the two anions, however, this remains to be tested in *planta*.

Acknowledgements: We thank Dr. Howitt (Australian National University) for kindly donating YSD1 and SHST1 and Patrick Loughlin for comments on the MS. This research was supported by The University of Adelaide and the Cooperative Research Centre for Viticulture.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.03.045.

References

[1] Smith, K.S., Balistrieri, L.S., Smith, S.M. and Severson, R.C. (1997) Distribution and mobility of molybdenum in the terrestrial environment in: Molybdenum in Agriculture (Gupta, U., Ed.), Cambridge University Press, Cambridge.
[2] Gupta, U. (1997) Soil and plant factors affecting molybdenum uptake in plants in: Molybdenum in Agriculture (Gupta, U., Ed.), pp. 71–91, Cambridge University Press, Cambridge.
[3] Mendel, R.R. and Hansch, R. (2002) Molybdoenzymes and molybdenum cofactor in plants. J. Exp. Bot. 53, 1689–1698.
[4] Kaiser, B.N., Gridley, K.L., Ngaire Brady, J., Phillips, T. and Tyerman, S.D. (2005) The role of molybdenum in agricultural plant production. Ann. Bot. Lond. 96, 745–754.
[5] Self, W.T., Grunden, A.M., Hasona, A. and Shanmugam, K.T. (2001) Molybdate transport. Res. Microbiol. 152, 311–321.
[6] Tomatsu, H., Takano, J., Takahashi, H., Watanabe-Takahashi, A., Shibagaki, N. and Fujiwara, T. (2007) An *Arabidopsis thaliana* high-affinity molybdate transporter required for efficient uptake of molybdate from soil. Proc. Natl. Acad. Sci. USA 104, 18807–18812.
[7] Tejada-Jimenez, M., Llamas, A., Sanz-Luque, E., Galvan, A. and Fernandez, E. (2007) A high-affinity molybdate transporter in eukaryotes. Proc. Natl. Acad. Sci. USA 104, 20126–20130.
[8] Baxter, I. et al. (2008) Variation in molybdenum content across broadly distributed populations of *Arabidopsis thaliana* is controlled by a mitochondrial molybdenum transporter (MOT1). PLoS Genet. 4, e1000004.
[9] Buchner, P., Takahashi, H. and Hawkesford, M.J. (2004) Plant sulphate transporters: co-ordination of uptake, intracellular and long distance transport. J. Exp. Bot. 55, 1765–1773.
[10] Marschner, H. (1995) Mineral Nutrition of Higher Plants, Academic Press, New York.
[11] Mendel, R. and Schwarz, G. (1999) Molybdoenzymes and molybdenum cofactor in plants. Crit. Rev. Plant Sci. 18, 33–69.
[12] Dudev, T. and Lim, C. (2004) Oxyanion selectivity in sulfate and molybdate transport proteins: an ab initio/CDM study. J. Am. Chem. Soc. 126, 10296–10305.
[13] Alhendawi, R.A., Kirkby, E.A. and Pilbeam, D.J. (2005) Evidence that sulfur deficiency enhances molybdenum transport in xylem sap of tomato plants. J. Plant Nutr. 28, 1347–1353.
[14] Macleod, J.A., Gupta, U.C. and Stanfield, B. (1997) Molybdenum and sulfur relationships in: Molybdenum in Agriculture (Gupta, U., Ed.), pp. 229–244, Cambridge University Press, Cambridge.
[15] Smith, F.W., Ealing, P.M., Hawkesford, M.J. and Clarkson, D.T. (1995) Plant members of sulfate transporters reveal functional subtypes. Proc. Natl. Acad. Sci. USA 92, 9373–9377.
[16] Smith, F.W., Hawkesford, M.J., Prosser, I.M. and Clarkson, D.T. (1995) Isolation of a cDNA from *Saccharomyces cerevisiae* that encodes a high affinity sulphate transporter at the plasma membrane. Mol. Gen. Genet. 247, 709–715.
[17] Dubois, E. and Grenson, M. (1979) Methylamine/ammonia uptake systems in *Saccharmyces cerevisiae*. Mol. Gen. Genet. 175, 67–76.
[18] Schneider, K., Muller, A., Johannes, K., Diemann, E. and Kottmann, J. (1991) Selective removal of molybdenum traces from growth media of N_2-fixing bacteria. Anal. Biochem. 193, 292–298.
[19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol measurement. J. Biol. Chem. 193, 265–275.
[20] Tweedi, J.W. and Segel (1970) Specificity of transport process for sulfur, selenium and molybdenum anions by filamentous fungi. Biochim. Biophys. Acta 196, 95–106.
[21] Miyauchi, S. et al. (2006) Functional characteristics of NaS2, a placenta-specific Na^+-coupled transporter for sulfate and oxyanions of the micronutrients selenium and chronium. Placenta 27, 550–559.
[22] Llamas, A., Kalakoutskii, K.L. and Fernandez, E. (2000) Molybdenum cofactor amounts in *Chlamydomonas reinhardtii* depend on the Nit5 gene function related to molybdate transport. Plant Cell Environ. 23, 1247–1255.
[23] Cuppoletti, J. and Segel, H. (1974) Transinhibition kinetics of the sulfate transport system of *Penicillium notatum*: analysis based on an Iso Uni Uni velocity equation. J. Membr. Biol. 17, 239–252.
[24] Dawson, P.A., Pirlo, K.J., Steane, S.E., Nguyen, K.A., Kunzelmann, K., Chien, Y.J. and Markovich, D. (2005) The rat Na^+-sulfate cotransporter rNaS2: functional characterization, tissue distribution and gene (*scl13a4*) structure. Pflug. Arch. 450, 262–268.
[25] Stout, P.R., Meagher, W.R., Pearson, G.A. and Johnson, C.M. (1951) Molybdenum nutrition of crop plants. Plant Soil III 1, 51–87.
[26] Kannan, S. and Ramani, S. (1978) Studies on molybdenum absorption and transport in bean and rice. Plant Physiol. 62, 179–181.